Chryseobacterium piscicola sp. nov., isolated from diseased salmonid fish

Pedro Ilardi, Jorge Fernández and Ruben Avendaño-Herrera

Laboratorio de Veterinaria, Camino Melipilla 5641, Cerrillos, Santiago, Chile

Correspondence

Ruben Avendaño-Herrera
reavendano@yahoo.com

Eight bacterial strains isolated from diseased rainbow trout ($n=5$) and Atlantic salmon ($n=3$) were characterized by phenotypic and molecular taxonomic methods. The isolates were negative for the Gram-reaction, non-motile, rod-shaped and catalase- and oxidase-positive. Colonies on solid media were yellow, smooth, shiny and circular with regular edges. Growth occurred at 4–28 °C (optimum, 15 °C) and with 0–3 % NaCl (optimum, 0.5 %). Analysis of the 16S rRNA gene sequence allocated the micro-organisms to the genus *Chryseobacterium*, with *Chryseobacterium soldanellicola* PSD1-4^T and *Chryseobacterium soli* JS6-6^T as their closest relatives (96.9 and 97.1 % sequence similarity, respectively). The levels of DNA–DNA hybridization towards these nearest phylogenetic neighbours were below 17.1 %. The DNA G + C contents of strains VQ-6316s^T and VQ-4836s were 32.5 and 32.3 mol%, respectively. The predominant menaquinone was MK-6 and the major fatty acids were iso-C_{15:0}, anteiso-C_{15:0}, iso-C_{17:1ω9c}, iso-C_{17:0} 3-OH and summed feature 3 (comprising C_{16:1ω7c} and/or C_{16:1ω7t} and/or iso-C_{15:0} 2-OH). The eight isolates were classified as representatives of a novel species, *Chryseobacterium piscicola* sp. nov., with strain VQ-6316s^T (=CECT 7357^T=DSM 21068^T) as the type strain.

The family *Flavobacteriaceae* represents the main bacterial lineage in the phylum ‘*Bacteroidetes*’ (formerly the *Cytophaga–Flavobacterium–Bacteroides* group) (Bernardet *et al.*, 1996, 2002). Within this family, the genera *Bergeyella*, *Chryseobacterium* and *Riemerella* form a separate branch on the basis of rRNA cistron similarity studies and phenotypic characterization (Vandamme *et al.*, 1994). At the time of writing, the genus *Chryseobacterium* comprised 38 species, some of which are pathogenic to humans and animals (Bernardet *et al.*, 2002, 2005, 2006).

During the characterization of bacteria isolated from external lesions of diseased farmed Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) in Osorno, Chile, eight yellow-pigmented strains were recovered on modified Anacker and Ordal’s agar (AOA; 0.5 % tryptone, 0.05 % yeast extract, 0.02 % beef extract, 0.02 % sodium acetate, pH 7.2; Anacker & Ordal, 1955) incubated at 15 °C. Five strains were isolated from rainbow trout (VQ-106r, VQ-5916r, VQ-5946r, VQ-5926r, VQ-5966r) and the remaining three isolates (VQ-2206s, VQ-4836s, VQ-6316s^T) were recovered from Atlantic salmon. Strains were routinely cultured aerobically on AOA at 15 °C for 3 days. Stock cultures were preserved at –80 °C

in Criobilles tubes (AES Laboratory). The diseased fish showed the typical signs (i.e. skin and muscle ulcerative lesions on the flank and in the anus or peduncle area) observed in fish affected by *Flavobacterium psychrophilum*, the causative agent of bacterial cold-water disease and rainbow trout fry syndrome (Ilardi & Avendaño-Herrera, 2008). However, a comparative analysis of 16S rRNA gene sequences indicated that all the isolates were members of the genus *Chryseobacterium*. Although they were only retrieved from superficial lesions, not from internal organs, their virulence for fish has been demonstrated (Ilardi & Avendaño-Herrera, 2008). In order to determine the exact taxonomic position of the new isolates, a taxonomic study was performed using a polyphasic approach.

PCR amplification of the 16S rRNA genes of the eight isolates was conducted using universal primers 20F and 1500R (Weisburg *et al.*, 1991) and PCR products were purified using QIAquick PCR purification kits (Qiagen). The 16S rRNA gene sequence was determined directly using the PCR-amplified DNA as a sequencing template on an ABI PRISM 310 sequencer (Applied Biosystems) according to the manufacturer’s recommendations. The resulting sequences were compared with those available in GenBank, EMBL (<http://www.ebi.ac.uk>) and the Ribosomal Database Project using the program BLAST. Multiple alignments were performed using the CLUSTAL_X program (Thompson *et al.*, 1997) and a phylogenetic tree was constructed using the neighbour-joining method with MEGA3 software (Kumar *et al.*, 2004). All isolates formed a

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains VQ-106r, VQ-4836s and VQ-6316s^T are EU869188–EU869190, respectively.

An electron micrograph of cells of strain VQ-6316s^T and a table giving API ZYM profiles are available with the online version of this paper.

robust cluster within the genus *Chryseobacterium*. Data from sequence similarity analysis indicated that the closest relatives of the eight strains were *Chryseobacterium soldanellicola* PSD1-4^T and *Chryseobacterium soli* JS6-6^T, exhibiting 96.9 and 97.1% 16S rRNA gene sequence similarities, respectively (Fig. 1). Interestingly, when the sequence of strain VQ-6316s^T was compared with those of *Chryseobacterium* strains isolated from fish published by Bernardet *et al.* (2005), it shared nearly 100% sequence similarity with strains UOF CR4395 and UOF CR2995 (GenBank accession nos AY468455 and AY468454, respectively), isolated from Atlantic salmon in Finland.

DNA–DNA hybridization was performed as described by De Ley *et al.* (1970) with the modifications of Huß *et al.* (1983) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6×6 multicell changer and a temperature controller with *in situ* temperature probe (Varian). The levels of DNA–DNA relatedness between strain VQ-6316s^T and strain VQ-5926r, *C. soldanellicola* PSD1-4^T and *C. soli* JS6-6^T were 99.2, 17.1 and 13.2%, respectively, demonstrating that the isolates formed a tight group that was distinct from recognized species of the genus *Chryseobacterium*.

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between the fish isolates, other members of the genus *Chryseobacterium* and representatives of other related genera in the family *Flavobacteriaceae*. *Weeksellia virosa* ATCC 43766^T was used as an outgroup. Bootstrap values >50% are shown at nodes. Bar, 0.01 substitutions per nucleotide position.

Phenotypic characterization of the eight isolates was carried out as described by Bernardet *et al.* (2002) and according to standard methods (MacFaddin, 1980). The Gram reaction was tested by using the bioMérieux Gram-stain kit according to the manufacturer's instructions and the non-staining KOH method (Buck, 1982). Gliding motility was assessed by phase-contrast microscopy on a fresh Anacker and Ordal's broth culture by the hanging drop technique (Bernardet *et al.*, 2002). The presence of flexirubin-type pigments and cell wall-associated galactosamine glycans was assessed by adding 20 % KOH or 0.01 % Congo red (Sigma), respectively, to 3-day-old colonies (Bernardet *et al.*, 2002; Avendaño-Herrera *et al.*, 2004). Cytochrome oxidase activity (using *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; Sigma) and catalase production (using 3 % H₂O₂) were determined on glass slides. The following tests were performed according to MacFaddin (1980): oxidation/fermentation reactions, Voges–Proskauer reaction and arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase activities.

Hydrolysis of the following substrates was determined using AOA as the basal medium: gelatin (1 %), agar (3 %), starch (1 %), Tween 80 (1 %), casein (1 %), DNA (1 %), sheep blood (5 %) and tyrosine (0.1 %). Growth was tested at 0, 4, 15, 28, 37 and 42 °C on AOA. Growth in the presence of 0, 1, 1.5, 3 and 6 % (w/v) NaCl was also determined. Growth was tested on MacConkey, Simmons'

citrate, blood, R2A, marine 2216, nutrient and tryptone soy agars. Enzymic activities were assessed using API ZYM (bioMérieux) strips according to the manufacturer's instructions, with the exception that the incubation temperature was 15 °C.

The morphology of bacterial cells grown for 48 h on AOA was observed by light (1000× magnification) and transmission electron (100SX; JEOL) microscopy. Cells were prepared for electron microscopy as described by Spurr (1969) and Reynolds (1963).

The results of the phenotypic analyses of the eight isolates are given in the species description and in Table 1 and those from API ZYM tests are given in Supplementary Table S1 (available in IJSEM Online).

The respiratory quinones of strains VQ-6316s^T and VQ-4836s were analysed using reverse-phase HPLC by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). The major peak identified was that of menaquinone MK-6, which is consistent with that present in all other members of the family *Flavobacteriaceae*.

The fatty acids of strains VQ-6316s^T and VQ-4836s were extracted from cells grown on AOA at 15 °C for 48 h, methylated and analysed using the standard protocol of the Microbial Identification System (MIDI, Microbial ID) at

Table 1. Phenotypic characteristics that differentiate *Chryseobacterium piscicola* sp. nov. from closely related *Chryseobacterium* species

Taxa (the number of strains examined, when greater than 1, is given in parentheses): 1, *C. piscicola* sp. nov. (*n*=8); 2, *C. soldanellicola* PSD1-4^T (Park *et al.*, 2006); 3, *C. scopthalmum* (*n*=2); 4, *C. piscium* (*n*=4; de Beer *et al.*, 2006); 5, *C. balustinum* LMG 8329^T; 6, *C. soli* (*n*=2) (Weon *et al.*, 2008); 7, *C. indoltheticum* LMG 4025^T. Except where indicated otherwise, data for taxa 3, 5 and 6 are from Hugo *et al.* (2003). +, Positive; w, weakly positive; –, negative; v, variable; D, delayed; NA, no data available.

Characteristic	1	2	3	4	5	6	7
Growth on/at:							
MacConkey agar	–	–	–	–	+	+	+
5 °C	+	+	D	+	D	+	+
37 °C	–	+	–	–	+	v	–
Enzyme activities							
DNase	w	–	+	D	+	–	+
Urease	–	–	+	+	–	+	–
Nitrate reduction	–	–	–	+	+	–	–
Hydrolysis of:							
Starch	–	NA	–	–	–	+	–
Tween 80	–	–	+	v	+	+	+
Tyrosine	–	NA	+	–	–	+	+
Production of:							
H ₂ S	–	–	–	–	–	NA	+
Indole (Kovacs' reagent)	–	–	–	+	+	–	+
Alkaline reaction on Christensen's citrate	–	–	+	NA	–	NA	+
DNA G + C content (mol%)	32.5*	28.8*	34.2†	33.6*	33.1*	39.9*	33.8*

*Refers to type strain.

†Mean DNA G + C content calculated for six reference strains plus the type strain (data from Mudarris *et al.*, 1994).

the DSMZ as described by Sasser (1990). The predominant fatty acids (mean values) were: iso-C_{15:0}, 36.2%; anteiso-C_{15:0}, 13.9%; iso-C_{17:1}ω9c, 11.1%; iso-C_{17:0} 3-OH, 10.5%; and summed feature 3 (comprising C_{16:1}ω7c and/or C_{16:1}ω7t and/or iso-C_{15:0} 2-OH), 11.2%. Detailed fatty acid profiles of the novel isolates and their closest relatives are presented in Table 2.

Genomic DNA was purified following the method of Cashion *et al.* (1977) and the DNA G+C content was determined using the HPLC method according to Mesbah *et al.* (1989) by the DSMZ. The DNA G+C contents of strains VQ-6316s^T and VQ-4836s were 32.5 and 32.3 mol%, respectively. These values fall within the range (29–39 mol%) reported for members of the genus *Chryseobacterium* by Bernardet *et al.* (2006).

On the basis of the results obtained, the new isolates are sufficiently distinct from all recognized species of the genus *Chryseobacterium* to be assigned as representatives of a novel species of this genus, for which the name *Chryseobacterium piscicola* sp. nov. is proposed.

Description of *Chryseobacterium piscicola* sp. nov.

Chryseobacterium piscicola [pis.ci'cola. L. n. *piscis* fish; L. suff. *-cola* (from L. n. *incola*) inhabitant; N.L. n. *piscicola* an inhabitant of fish].

Cells are Gram-reaction-negative, non-motile rods approximately 1.6–3.5 µm in length and 0.8–1.2 µm in diameter (see Supplementary Fig. S1 available in IJSEM Online). Catalase- and oxidase-positive and non-fermentative. Colonies are smooth, shiny, circular with regular edges and yellow in colour. Flexirubin pigments are produced, but Congo red is not absorbed. Grows at 4–28 °C (optimum, 15 °C) and with 0–3 % NaCl (optimum, 0.5 %). Good growth occurs on blood (α-haemolytic), R2A, nutrient and tryptone soy agars, but no growth occurs on MacConkey, marine 2216 or Simmons' citrate agars. Gelatin and aesculin are hydrolysed, but Tween 80, tyrosine, agar, starch and casein are not. All strains are negative for arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase activities. DNA reaction is weakly positive. Nitrate and nitrite are not reduced. The methyl red and Voges-Proskauer tests are negative. In the API ZYM system, alkaline phosphatase, esterase, esterase lipase, lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-glucuronidase, α-glucosidase, β-glucosidase and *N*-acetyl-β-glucosaminidase activities are present, but trypsin, α-galactosidase, β-galactosidase, α-mannosidase and α-fucosidase activities are absent. The predominant menaquinone is MK-6 and the major (>10 %) fatty acids are iso-C_{15:0}, anteiso-C_{15:0}, iso-C_{17:1}ω9c, iso-C_{17:0} 3-OH and summed feature 3

Table 2. Cellular fatty acid content (%) of *Chryseobacterium piscicola* sp. nov. and closely related species of the genus *Chryseobacterium*

Taxa: 1, *C. piscicola* sp. nov. (*n*=2); 2, *C. soldanellicola* PSD1-4^T (Park *et al.*, 2006); 3, *C. scopthalmum* (*n*=2); 4, *C. piscium* (*n*=4; de Beer *et al.*, 2006); 5, *C. balustinum* LMG 8329^T; 6, *C. soli* JS6-6^T (Weon *et al.*, 2008); 7, *C. indoltheticum* LMG 4025^T. Data for taxa 3, 5 and 6 are taken from Hugo *et al.* (2003). Values derived from more than one strain are mean ± SD. ND, Not detected; NR, not reported; tr, trace (less than 1.0 %). Fatty acids amounting to <1 % of the total fatty acids in all strains studied are omitted. Different culture conditions were used.

Fatty acid	1	2	3	4	5	6	7
iso-C _{13:0}	2.4 ± 0.8	tr	tr	tr	tr	1.0	ND
Unknown 13.566*	1.3 ± 0.5	2.3	2.9 ± 0.2	tr	1.6	NR	1.7
iso-C _{15:0}	36.2 ± 0.2	41.8	35.0 ± 0.7	38.3 ± 5.0	32.3	15.7	29.4
anteiso-C _{15:0}	13.9 ± 0.0	1.9	tr	2.7 ± 1.9	tr	3.8	5.9
iso-C _{16:0}	1.0 ± 0.1	NR	NR	NR	NR	1.6	NR
C _{16:0}	2.0 ± 0.2	1.4	1.2 ± 0.2	1.1 ± 0.2	1.6	5.8	1.0
iso-C _{15:0} 3-OH	2.1 ± 0.0	2.7	2.7 ± 0.1	2.4 ± 0.3	2.7	2.4	2.3
iso-C _{17:1} ω9c	11.1 ± 0.2	14.6	24.8 ± 0.4	18.7 ± 2.8	27.1	15.5	25.6
Unknown 16.580*	tr	1.5	1.5 ± 0.5	1.2 ± 0.2	1.3	NR	1.3
iso-C _{17:0}	tr	tr	tr	1.2 ± 0.2	1.0	NR	tr
iso-C _{16:0} 3-OH	tr	tr	tr	tr	tr	2.1	1.3
C _{16:0} 3-OH	2.2 ± 0.1	1.3	1.0 ± 0.1	1.3 ± 0.3	1.4	2.2	tr
C _{18:1} ω5c	tr	tr	NR	NR	tr	1.1	tr
iso-C _{17:0} 3-OH	10.5 ± 0.1	17.7	16.3 ± 0.1	16.2 ± 3.1	16.8	15.0	14.0
C _{17:0} 2-OH	1.5 ± 0.0	tr	NR	NR	tr	2.3	3.0
Summed feature 3†	11.2 ± 0.5	ND	NR	10.8 ± 1.3	tr	12.0	NR

*Unknown fatty acid; numbers indicate the equivalent chain-length.

†Summed features are groups of two or three fatty acids that cannot be separated by GLC using MIDI systems. Summed feature 3 comprised C_{16:1}ω7c and/or C_{16:1}ω7t and/or iso-C_{15:0} 2-OH.

(comprising C_{16:1}ω7c and/or C_{16:1}ω7t and/or iso-C_{15:0} 2-OH).

The type strain, VQ-6316s^T (=CECT 7357^T=DSM 21068^T), was isolated from diseased Atlantic salmon (*Salmo salar*) farmed in Osorno, Chile. The DNA G+C content of the type strain is 32.5 mol%.

Acknowledgements

This work was supported by grant IPC 019 from the Program Bicentenario Ciencia y Tecnología, CONICYT, Chile. R.A.-H was also supported in part by this Grant. Thanks are expressed to members of the technical team of the laboratories of Veterquímica. The authors wish to express their gratitude to Dr J.-F. Bernardet, Institut National de la Recherche Agronomique, Unité de Virologie et Immunologie Moléculaires, for reviewing and editing the manuscript.

References

- Anacker, R. L. & Ordal, E. J. (1955). Study of a bacteriophage infecting the myxobacterium *Chondrococcus columnaris*. *J Bacteriol* **70**, 738–741.
- Avendaño-Herrera, R., Magariños, B., López-Romalde, S., Romalde, J. L. & Toranzo, A. E. (2004). Phenotypic characterization and description of two major O-serotypes in *Tenacibaculum maritimum* strains isolated from marine fishes. *Dis Aquat Organ* **58**, 1–8.
- Bernardet, J.-F., Segers, P., Vancanneyt, M., Berthe, F., Kersters, K. & Vandamme, P. (1996). Cutting a Gordian knot: emended classification and description of the genus *Flavobacterium*, emended description of the family *Flavobacteriaceae*, and proposal of *Flavobacterium hydatis* nom. nov. (basonym, *Cytophaga aquatilis* Strohl and Tait 1978). *Int J Syst Bacteriol* **46**, 128–148.
- Bernardet, J.-F., Nakagawa, Y. & Holmes, B. (2002). Proposed minimal standards for describing new taxa of the family *Flavobacteriaceae* and emended description of the family. *Int J Syst Evol Microbiol* **52**, 1049–1070.
- Bernardet, J.-F., Vancanneyt, M., Matte-Tailliez, O., Grisez, L., Tailliez, P., Bizet, C., Nowakowski, M., Kerouault, B. & Swings, J. (2005). Polyphasic study of *Chryseobacterium* strain isolated from diseased aquatic animals. *Syst Appl Microbiol* **28**, 640–660.
- Bernardet, J.-F., Hugo, C. & Bruun, B. (2006). The genera *Chryseobacterium* and *Elizabethkingia*. In *The Prokaryotes. A Handbook on the Biology of Bacteria*, 3rd edn, vol. 7, pp. 638–676. Edited by M. Dworkin, S. Falkow, E. Rosenberg, K. H. Schleifer & E. Stackebrandt. New York: Springer-Verlag.
- Buck, J. D. (1982). Nonstaining (KOH) method for determination of gram reactions of marine bacteria. *Appl Environ Microbiol* **44**, 992–993.
- Cashion, P., Holder-Franklin, M. A., McCully, J. & Franklin, M. (1977). A rapid method for base ratio determination of bacterial DNA. *Anal Biochem* **81**, 461–466.
- de Beer, H., Hugo, C. J., Jooste, P. J., Vancanneyt, M., Coenye, T. & Vandamme, P. (2006). *Chryseobacterium piscium* sp. nov., isolated from fish of the South Atlantic Ocean off South Africa. *Int J Syst Evol Microbiol* **56**, 1317–1322.
- De Ley, J., Cattoir, H. & Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133–142.
- Hugo, C. J., Segers, P., Hoste, B., Vancanneyt, M. & Kersters, K. (2003). *Chryseobacterium joostei* sp. nov., isolated from the dairy environment. *Int J Syst Evol Microbiol* **53**, 771–777.
- HuB, V. A. R., Festl, H. & Schleifer, K. H. (1983). Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. *Syst Appl Microbiol* **4**, 184–192.
- Ilardi, P. & Avendaño-Herrera, R. (2008). Isolation of *Flavobacterium*-like bacteria from diseased salmonids cultured in Chile. *Bull Eur Assoc Fish Pathol* **28**, 176–185.
- Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for molecular evolutionary genetic analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.
- MacFaddin, J. F. (1980). *Biochemical Tests for Identification of Medical Bacteria*, 2nd edn. Baltimore, MD: Williams & Wilkins.
- Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Mudarris, M., Austin, B., Segers, P., Vancanneyt, M., Hoste, B. & Bernardet, J.-F. (1994). *Flavobacterium scophthalmum* sp. nov., a pathogen of turbot (*Scophthalmus maximus* L.). *Int J Syst Bacteriol* **44**, 447–453.
- Park, M. S., Jung, S. R., Lee, K. H., Lee, M.-S., Do, J. O., Kim, S. B. & Bae, K. B. (2006). *Chryseobacterium soldanellicola* sp. nov. and *Chryseobacterium taeanense* sp. nov., isolated from roots of sand-dune plants. *Int J Syst Evol Microbiol* **56**, 433–438.
- Reynolds, E. S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J Cell Biol* **17**, 208–212.
- Sasser, M. (1990). *Identification of bacteria by gas chromatography of cellular fatty acids*, MIDI Technical Note 101. Newark, DE: MIDI Inc.
- Spurr, A. R. (1969). A low-viscosity epoxy resin embedding medium for electron microscopy. *J Ultrastruct Res* **26**, 31–43.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.
- Vandamme, P., Bernardet, J.-F., Segers, P., Kersters, K. & Holmes, B. (1994). New perspectives in the classification of the flavobacteria: description of *Chryseobacterium* gen. nov., *Bergeyella* gen. nov., and *Empedobacter* nom. rev. *Int J Syst Bacteriol* **44**, 827–831.
- Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 697–703.
- Weon, H.-Y., Kim, B.-Y., Yoo, S.-H., Kwon, S.-W., Stackebrandt, E. & Go, S.-J. (2008). *Chryseobacterium soli* sp. nov. and *Chryseobacterium jejuense* sp. nov., isolated from soil samples from Jeju, Korea. *Int J Syst Evol Microbiol* **58**, 470–473.